Date Palm Tissue Culture

U.S. Department of Agriculture
Agricultural Research Service
Advances in Agricultural Technology • AAT-W-17/August 1981

ACKNOWLEDGMENTS

This study is the culmination of research conducted while the author was at the U.S. Date and Citrus Station, Indio, Calif., between June 1977 and May 1980. Grateful acknowledgment is made to collaborators: A. M. Torres, University of Kansas, Lawrence, for electrophoresis work; D. A. DeMason and J. I. Stillman, University of California, Riverside, for histological studies; B. J. Finkle and J. M. Ulrich, Western Regional Research Center, Albany, Calif., for cryogenic research; and D. W. Ramming, U.S. Horticultural Station, Fresno, Calif., for helpful advice and criticism in the preparation of this paper.

A copy of this publication is available upon request from Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, CA 91106.

International Standard Serial Number (ISSN) 0193-3736

Agricultural Research Service, Advances in Agricultural Technology, Western Series, No. 17, August 1981

Published by Agricultural Research Service (Western Region), U.S. Department of Agriculture, Oakland, Calif. 94612

ABSTRACT

This report is concerned with the rapid propagation of the date palm (*Phoenix dactylifera* L.) *in vitro* and the physical and chemical factors of the environment in which it occurs. Ten species of the Palmae have been introduced to *in vitro* conditions to date. Of these 10 species, 5 have produced embryogenetic callus and 2 have produced free-living plantlets from callus. A discussion of these reports is provided.

A procedure to rapidly propagate date palm through tissue culture is presented. Adventitious plantlets may be obtained from lateral buds, shoot tips, embryos, and pieces of stem and rachilla tissue cultured on a modified Murashige and Skoog nutrient medium containing 3 mg/L N-(Δ^2-isopentyl)adenine, 100 mg/L 2,4-dichlorophenoxyacetic acid, and 3 g/L activated charcoal. Additions of auxins were necessary to induce calli, adventitious plantlets, and roots. Addition of activated charcoal was essential to foster satisfactory by reducing explant browning.

Date palm plantlets arise through an asexual embryogenesis process. Comparative studies revealed basic similarities between zygotic and asexual embryos in several stages of development.

A procedure to successfully transfer plantlets from *in vitro* to free-living conditions is presented. Attempts to obtain a quality control test to verify the clonal nature of tissue cultured plantlets is described using gene-enzyme systems and morphological features. Cryostorage of date palm callus at -196°C and its recovery are discussed as a means of future palm germplasm preservation.

KEYWORDS: Cryostorage, palm tissue culture, *Phoenix dactylifera* L., tissue culture procedures.

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By Brent Tisserat[1]

INTRODUCTION

Progress in breeding, genetics, crop improvement, and expansion of commercial plantings of date palm (*Phoenix dactylifera* L.) has been restricted by slow asexual propagation, which employs only the clonal offshoots derived from axillary buds on the palm. Progagation by seed is unsatisfactory because this species is dioecious and completely heterozygous. Seedlings are about equally divided between male and female plants, and the seedlings are not true to type. By using only offshoots, new or scarce varieties, and disease or pest-resistant varieties, of date palm cannot be propagated quickly and distributed on a large scale (*36*, *53*).[2] This report consolidates information of date palm tissue culture and provides a method to rapidly propagate date palms *in vitro* (under artificial conditions) and to grow the plants under free-living conditions.

LITERATURE REVIEW

General Remarks

Development of techniques for successful tissue culture of the date palm has required intensive use of existing information on work done on other crops (*10*, *33*, *49*) and especially on the commercially important coconut palm (*Cocos nucifera* L.) (*4*, *18*) and oil palm (*Elaeis guineensis* Jacq.) (*6*, *29*), which have no natural means of asexual propagation. Because of current interest, I have included a broad review of tissue culture techniques used in developing micropropagation of various species in the Palmae, a family in which axillary branching is an infrequent species habit (*6*, *9*, *27*, *30*, *36*, *37*, *51*, *67*).

Nutrient medium supplemented with coconut water (CW) from green coconut fruit has been reported to enhance the survival and growth of a wide spectrum of plant cells, tissues, and organs cultured *in vitro* (*63*, *70*). In the first report of palm tissue culture, Cutter and Wilson (*7*) discussed excised coconut embryos developed on a nutrient medium containing liquid filter-sterilized coconut endosperm.

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[2]Italic numbers in parentheses refer to Literature Cited, p. 24.

The observations of later investigators are compiled in table 1, dealing with the family Palmae. Most of these investigations have been performed since 1970. The production of plantlets and asexual embryos has been reported in date and oil palms. In most of these cases, explant tissues produced callus that gives rise to asexual embryos. These asexual embryos germinate and developed into plantlets through a sequence of development analogous to the events occurring within a seed. A few instances of plantlets produced from rooted growing tips have also been reported (*53*, *62*, *67*). Embryo culture is a specialized type of tissue culture in which excised embryos are removed from the seed and germinated *in vitro*. In such cases, vegetative propagation is not obtained. This specialized type of tissue culture is employed to study sequences of embryonic development or to help to insure survival of unique crosses. Embryo culture studies are only briefly mentioned in this review.

Oil Palm

The earliest reports of the production of organized structures from calli and plantlets from palm tissue cultures were made in the oil palm (*28*, *31*, *41*, *48*, *61*, *62*). Staritsky (*62*) cultured apical tips and induced leaf and root development. Dissected 2-year-old shoot tips produced juvenile pinnate leaves and adventitious roots on a modified Miller's basal medium.

Rabechault et al. (*41*) reported that embryo callus cultured in a liquid medium containing CW, indole-3-acetic acid (IAA), kinetin, 2,4-dichlorophenoxyacetic acid (2,4-D), and 20 percent sucrose, produced embryolike structures. Smith and Jones (*60*) also reported similar observations with embryo callus. Later investigators confirmed these initial reports (*28*, *29*, *61*). Embryolike structures from callus developed from clonal tissues including apical tips, leaf bases, and roots (*28*, *29*, *32*, *49*). Corley et al. (*6*) reported the successful introduction of tissue cultured oil palms into free-living plantation conditions.

Coconut Palm

Numerous attempts to successfully culture excised embryos from coconuts *in vitro* have been reported (*2*, *5*, *7*, *11*, *21*, *22*, *57*, *75*). Eeuweens (*16*) obtained calli from inflorescence, leaf, and stem tissues by using a nutrient medium supplemented with additional iodine and containing gibberellic acid, kinetin, and 2,4-D. Production of roots from petiole and rachilla tissues has been reported (*17*, *18*).

Coconut inflorescences in culture were stimulated to produce meristematic spikelike projections composed of a succession of leaves. Rooting of these structures was not achieved. Initial callus production has been obtained from a variety of coconut explants, but further subculturing was not possible (*16*, *17*, *18*). Subcultural callus obtained from endosperm tissues and excised embryos has been reported (*14*, *18*, *21*). Structures resembling initial embryogenic stages have been observed in endosperm (*21*) and stem (*4*) callus. Coconut plantlets have not been obtained to date.

Table 1.--*Tissue culture in the Palmae*

Plant	Explant	Growth response	Reference
Caryota urens L.	Zygotic embryo	Germination	77
Chamaedorea costaricana Oerst.	--do--	Callus/asexual embryos	54, 55
Cocos nucifera L.	Inflorescence	Callus/roots	16, 17, 18
	Leaf/petiole	--do--	16, 17
	Roots	Lateral root initiation	23
	Stem	Callus	4, 16, 17, 18
	Zygotic embryo	Germination	2, 5, 7, 11, 12, 13, 21, 22, 57, 75
Elaeis guineensis Jacq.	Apical tip	Callus/asexual embryos	6, 28, 29, 48, 61
	--do--	Leaf and root initiation	62
	Inflorescence	Flower development	1
	--do--	Callus/asexual embryos	6, 28, 29
	Root	Root elongation/callus	31, 32
	--do--	Callus/asexual embryos	6, 28, 29, 61
	Zygotic embryo	--do--	29, 41, 60, 61
	--do--	Germination	38, 39, 40, 42, 43, 44, 45, 46, 47
Howeia forsteriana Becc.	--do--	Callus/asexual embryos	54, 55
Mascarena lagenicaulis L.	--do--	Germination	77
Mascarena vershaffeltii L.	--do--	--do--	77
Phoenix dactylifera L.	Apical tip and lateral buds	Leaf differentiation	19, 36, 51, 58, 67, 68
	--do--	Leaf differentiation/ root initiation	36, 51, 52, 53, 56, 71
	--do--	Callus	36, 51, 52, 53
	--do--	Callus/asexual embryos	67, 68, 69, 71
	Inflorescences	--do--	54, 55, 67, 71
	Meristem	--do--	54, 55, 67, 71
	Petiole	Callus/roots	18
	Polyembryonic embryos	Callus/asexual embryos	50
	Root	Plantlet initiation	60

Table 1.--*Tissue culture in the Palmae*--Continued

Plant	Explant	Growth response	Reference
	Seed	Callus/asexual embryos	3
	Zygotic embryo	--do--	54, 55, 67, 68, 71
	--do--	Germination	36, 51, 52, 53, 67, 69
Pritchardia kaalae Rock	--do--	--do--	26
Veitchia joannis H. Wendl.	--do--	--do--	26

Date Palm

Reuveni and his colleagues in Israel conducted pioneer tissue culture research with the data palm (*36*, *51*, *52*, *53*). Several explant sources and media were used to obtain morphogenetic responses *in vitro*. Browning of the medium and the explant was a frequent and prominent problem, which resulted in premature explant death. In some instances, growing tips produced roots, and some callus was induced from explants on medium containing various concentrations of kinetin and napthaleneacetic acid (NAA). This callus was short lived and could not be subcultured (*51*, *53*).

Schroeder (*58*) observed that excised growing tips and lateral buds dissected from offshoots cultured *in vitro* enlarged considerably and initiated leaves. Seedling root tips excised from germinating seeds have been reported to give rise to free-living plants (*59*). Germinating seeds cultured on a Knob's basal medium supplemented with CW, NAA, and kinetin produced callus from the cotyledonary sheath region, which gave rise to buds and roots (*3*). Excised polyembryonic embryos--cultured on a nutrient medium containing casien hydrolyzate, kinetin, indole-3-butyric acid (IBA), NAA, 2,4-D, charcoal--produced calli and asexual embryos (*50*).

Callus and asexual embryos have been produced from excised zygotic embryos and young inflorescences on a nutrient medium containing charcoal and high auxin concentrations, for example, 30 and 100 mg/L 2,4-D (*54*, *55*, *67*). Production of callus, asexual embryos, and plantlets from other clonal tissues--including lateral buds, meristem and shoot tips--has also recently been reported (*67*, *68*, *69*, *71*). Free-living plants have been established in soil from these plantlets (*67*). Propagation of the date palm through division and rooting of lateral buds and shoot tips has been investigated (*37*, *51*, *56*, *71*).

Other Palm Species

Embryo culture of several ornamental palm species has been reported in *Caryota urens* L., *Mascarena lagenicaulis* L., *M. vershaffeltii* L., *Pritchardia kaalae* Rock, and *Veitchia joannis* H. Wendl. (*26*, *77*).

Charcoal enhanced the germination and subsequent development of excised embryos (*77*). Applications of high auxin concentrations to nutrient medium containing charcoal resulted in formation of embryogenic callus from immature excised embryos of *Chamaedorea costaricana* Oerst. and *Howeia forsteriana* Becc. (*54*, *55*).

Cultural and Physiological Factors in Tissue Culture

Nutrient Media

Nutrient medium employed in palm tissue culture must satisfy two prerequisites: (*1*) provide the basic nutrients necessary for explant survival, and (*2*) prevent or retard browning of the explant and the medium.

Composition.--Callus initiation and its subsequent survival and proliferation have been achieved in palms using a variety of media (*3*, *4*, *28*, *52*, *63*). Eeuweens (*16*) found that addition of supplemental iodine enhanced coconut callus production. The ratio of ammonium to nitrates was critical to coconut callus development (*4*, *17*). Reuveni (*50*) employed half-strength Murashige and Skoog macrosalts to produce callus from excised date embryos. Other investigators, however, have been able to induce embryogenetic callus and morphogenetic responses from palm tissues using common, unaltered nutrient media (*3*, *28*, *55*, *56*, *67*).

Addition of complex addenda, such as CW, casien hydrolysate, malt extract, and other undefined nutrients, to culture media to enhance palm tissue growth has been reported (*3*, *4*, *16*, *17*, *53*, *61*) but is not required for growing palm tissue *in vitro*. Other investigators have been able to induce prolific callus production and plantlet development from palm tissues on chemically defined media (*53*, *67*, *68*). A suitable nutrient medium to obtain callus production from palm tissues should have a balanced ratio of sugars, salts, and vitamins and should be supplemented with auxins.

The concentration of hormones, especially auxins, is the most critical factor in stimulating callus production and subsequent embryogenesis (*54*, *55*, *67*). Addition of cytokinins is marginally beneficial to callus production.

Adsorbents.--Browning is a wound response, occurring when the explant is cut (*21*, *53*). The inhibitory effect of explant and media browning has been frequently reported in palm tissue cultures (*21*, *53*, *61*, *67*, *77*). Smith and Thomas (*61*) advocated excision of browning explant parts during culture to prevent this problem. Adsorbents have been applied to palm tissue cultures with promising results (*21*, *50*, *51*, *52*, *53*, *55*, *67*, *77*). Reuveni and Lilien-Kipnis (*52*, *53*) noted that addition of charcoal to nutrient medium enhanced the development of date palm tips. Application of charcoal to nutrient medium has been reported to reduce browning of excised palm embryos (*21*, *76*).

Addition of other adsorbents to nutrient medium, such as ascorbic acid, dihydroxynaphthalene, dimethyl sulfoxide, and polyvinylpyrrolidone, were ineffective against browning in date palm explants (*53*, *67*). Rhiss et al. (*56*), however, found that addition of a combination of adsorbents, including ammonium citrate, adenine, glutamine, and polyvinylpyrrolidone, successfully retarded browning of bud and tip date palm explants. Apavatjrut and Blake (*4*) suggested that browning could be eliminated by using a nutritionally balanced medium.

Explant Factors

Explant source.--The palm tree and its offshoots have a number of parts from which explants can be selected (table 2). Production of callus and plantlets from almost all parts of the palm tree has been reported. Generally, the older and more differentiated tissues and organs have less of a chance to undergo morphogenesis *in vitro* than younger, more meristematic tissues (*58*, *63*). Explant sources procured from leaves, petioles, rachillae, and roots are of little value in tissue culture. Conversely, meristematic tissues from immature organs, growing tips, and embryos would be the tissues of choice to culture *in vitro* (*55*, *61*, *67*).

Table 2.--*Source of palm explants and their morphogenetic potential* in vitro

Explant source	Availability	Morphogenetic potential	Remarks
Apical tip	One per plant[1]	High	Must sacrifice plant to obtain.
Influorescence	Abundant	Variable	Seasonal; immature--high; mature--low morphogenetic potential.
Lateral buds	Several	--do--	Species specific; must sacrifice plant to obtain; type and degree of differentiation related to morphogenetic potential.
Leaf	Abundant	Low	Highly differentiated structure.
Meristem	--do--	--do--	Must sacrifice plant to obtain.
Petiole	--do--	--do--	Highly differentiated structure.
Rachis	--do--	--do--	Seasonal; highly differentiated structure.
Root	--do--	Variable	Tips only have high morphogenetic potential; notable contamination problems.
Zygotic embryo	--do--	--do--	Not clonal; immature embryos have a higher morphogenetic potential than older embryos.

[1] Tree or offshoot.

Much work in palm tissue culture has been performed using excised embryos (*3*, *42*, *53*); however, clonal tissues excised from meristematic regions of old, established varieties have also been shown to possess a high morphogenetic potential (*28*, *55*, *67*).

Shoot tips may be used to vegetatively propagate desired palm clones. Usually, only one shoot tip per palm occurs, and the tree must be sacrificed to obtain this tip. Lateral buds are numerous in the date palm, but only a few viable buds can be obtained from each palm and/or its offshoots. Usually, only those buds with prior leaf development and that are meristematically active can survive in culture (*52*, *67*, *71*). Production of oil palms from root tips has been reported (*28*, *29*).

Surface sterilization.--The initial explant must be adequately disinfected and rendered free of contaminants for successful tissue cultures to be produced. Contaminants have been found to be a considerable problem to overcome in palm cultures (*21*, *51*, *61*, *67*). The disinfection procedure used depends on the original condition of the explant. The most common surface sterilization procedure involves submergence of the explant in a sodium hypochlorite solution (0.26 to 2.6 percent) with a few drops of emulsifier for 15 to 30 minutes. Before planting, the explant is usually rinsed several times with sterile distilled water to remove residual disinfectant (*16*, *53*, *55*, *67*, *68*). Following aseptic preparation of the explant, a further dip for 5 to 10 seconds in sodium hypoclorite solution, before planting on the medium, may be advantageous (*67*).

Other chemical sterilants, such as chloramine-T, 8-hydroxguiinoline, peracetic acid, and mercury chloride, have been used without success to disinfect palm tissues (*61*). Alcohol soak and flame treatments have been used as disinfectants (*36*, *53*, *58*, *61*). Uncontaminated coconut inflorescences may be obtained from unopened spathes without using chemical sterilants (*16*, *17*). The occurrence of contamination days, weeks, or even months following surface sterilization of explants is not uncommon (*7*, *21*, *61*). Internal contaminants usually do not reveal themselves for some time following inoculation to sterile medium; this is especially the case for root cultures. The addition of antibiotics, such as penicillin-streptomycin or gentamicine, has been advocated (*21*, *61*); however, Reuveni and Lilien-Kipnis (*53*) found that addition of antibiotics was ineffective against date palm contaminants.

Transfer of Plantlets to Free-Living Conditions

Transfer of tissue cultured plantlets to free-living conditions is a critical step in micropropagation. Plantlets must have well-developed root and shoot systems to satisfy their photosynthetic and transpiration requirements. Plantlets should be kept under high humidity conditions to minimize transpiration until they are suitably acclimated. Free-living plantlets have been obtained from germinated excised embryos (*26*, *58*, *77*). Tissue cultured palms benefited from an antitranspiration treatment and partial defoliation during establishment in free-living conditions (*6*, *37*, *56*, *67*).

Genetic variation among plants propagated through tissue culture from callus is possible due to variation in chromosome number, for example, loss or addition of chromosomes, and gene mutation, or a combination of these factors (*8*, *15*, *25*, *34*).

To determine the clonal nature of the tissue cultured palms, the plantlets should be grown to an early bearing stage and its vegetative and fruiting characteristics compared with those of the parent variety. Preliminary quality control tests to indicate that tissue cultured palms are genetically uniform may include: chromosome counts, phenotypic examination of vegetative growth, and variation in isozymes of gene-enzyme systems (*25*). Palm chromosomes are numerous and small compared with those of most other plants; mitotic examination of tissue cultured palms are unreliable (*6*). Alternatively, the use of isozymes of various gene-enzyme systems may help determine genetic sports and mutations produced from palm cultures (*72*, *75*).

Morphogenetic Responses

Plantlets Derived From Callus

Callus produced from excised oil palm embryos has been reported to give rise to asexual embryos (*29*, *41*, *60*, *61*). Embryogenetic callus was composed of a variety of cellular structures. Proembryos were identified as pearly white globular structures with a well-defined epidermal layer. These early asexual embryos resembled the zygotic embryo by containing storage protein and lipid bodies (*28*, *41*). The embryonic precursors elongated and acquired polarity. Eventually, these embryos germinated and became plantlets (*28*).

In date palm, embryogenetic callus derived from clonal tissues was found to be a heterogenous composition, consisting of different cell types and structures (*55*, *69*). Callus produced from explants on medium containing 2,4-D was composed of numerous proembryonic precursors or meristematic centers. Single cells were observed to divide and give rise to proembryonic meristematic compact centers within the callus (*69*). These meristematic loci developed into bipolar embryos with a meristematic root-shoot end and less meristmatic haustorial end. The root-pole end of the embryo remained embedded within the callus mass while the haustorium protruded outwards. Transfer to medium devoid of 2,4-D allowed these bipolar embryos to develop further and undergo asexual embryogenesis to produce a plantlet with a distinct shoot and root system. The haustorium of the asexual embryo was vestigial. The zygotic and asexual embryogenetic events were found to be quite similar.

Coconut callus, unlike that of the embryogenetic oil or date palm callus, has been characterized as being distinct. Coconut explants from mature tissues were found to produce callus that cannot be subcultured further (*4*, *16*, *17*, *18*). This coconut callus has a definite organization; cells are arranged in ranks and terminate in loose chains (*18*). Histological examination of subcultural endosperm callus revealed it was composed of undifferentiated parenchyma cells and scattered compact meristematic loci (*21*). Some early proembryonic structures have been observed to develop in coconut callus, but later differentiation was not obtained (*4*, *20*).

Plantlets Derived From Growing Tips and Lateral Buds

The production of plantlets through the rooting of shoot tips and lateral buds has been reported in both oil (*62*) and date palms (*37*, *52*, *53*, *56*, *71*).

Control of the production of roots from shoot tips has been reported in date palms (*37*, *56*). This type of micropropagation is analagous to rooting of miniature offshoots *in vitro*.

Plantlets Derived From Embryo Cultures

The initial size and developmental stage of the excised embryo are critical factors to obtaining viable plantlets using tissue culture. Older embryos have a greater chance to germinate and produce plants than younger, less differentiated embryos. The nutrient medium used to culture excised embryos must satisfy the nutritional contribution of the seed endosperm. Embryogensis *in vitro* has been found to recapitulate the events found in the seed (*69*).

Potential Applications of Tissue Culture

Only tissue cultured plants are currently used in the commercial propagation of the oil palm (*6*). The success of propagating date palm tissue remains to be determined, but the work with oil palms indicates that other palms may be similarly propagated. Research is still needed to improve commercial application techniques and to develop suitable quality control tests to verify the clonal nature of callus-derived plantlets. Palm tissue culture research should also include developing methods (1) to mass produce palms from callus using an assembly line system and (2) to master the mechanism of division and rooting of lateral buds *in vitro*.

Potentially, tissue culture of palms would allow: (1) clones of beneficial F_1 hybrids, disease-resistant palms, and previously select and seed-only-originated palms to be vegetatively propagated, and (2) large numbers of genetically uniform palms to be distributed. Currently, both the coconut and the date palm industries are plagued by epidemic diseases--lethal yellowing and bayoud, respectively. Micropropagation should find a ready application in the coconut, date, and oil palm industries as well as in the production of desired ornamental palms.

In addition to its rapid propagation advantages, tissue culture techniques may be used with other scientific disciplines as a useful tool. Gradual reduction of plant germplasm has been a common occurrance in the 20th century (*79*). Micropropagation may be coupled with a cryogenetic program to conserve rare or endangered palm genotypes for future use. Germplasm preservation programs with palms are expensive, labor intensive, and often impractical. For example, most palms cannot be vegetatively propagated by natural means. Preliminary studies have indicated that date palm callus may be stored at low temperatures with minimum cost and maintenance requirements (*20*, *73*). Revived callus produced plantlets normally. Genetic engineering studies in palms using tissue culture techniques, such as protoplast fusion and anther culture, may rapidly advance palm breeding programs (*18*).

Palms development and vegetative propagation potential are poorly understood but economically important in tropical and subtropical regions. Hopefully, improved methods to propagate and study the trees in the family Palmae will result in more economic use of these plants.

PROCEDURE TO TISSUE CULTURE THE DATE PALM

Laboratory Preparation

Preparation, construction, and staffing of a tissue culture laboratory should be undertaken with a specific goal and should employ proven methods. When success in a specific project has been demonstrated, branching into new fields may be pursued. Initial projects, successfully completed, should lay a foundation of confidence, experience, and skill necessary for further work.

Reference publications concerned with tissue culture techniques and laboratory preparations are available (*10*, *24*, *33*, *64*, *66*, *78*). The purpose of this section is to add appropriate remarks to these references.

Plant tissue culture laboratories, whether devoted to scientific research or commercial vegetative propagation, should contain a number of common essential facilities (*10*, *64*). General diagrammatic layouts for a tissue culture laboratory are presented in figures 1 and 2. All tissue culture laboratories should have separate areas that include: a dishwashing area, to clean and store glassware; a general laboratory devoted to explant and medium preparations and associated activities; a transfer room, in which to inoculate sterile medium with explants and to conduct aseptic transferring; and a culture room where cultures are incubated. These facilities must be planned within the limits of sanitation, structural accomodations, budget, and staff.

The laboratory and the culture room must have rigidly controlled temperature ranges. A suitable central heat and air-conditioning system should suffice. Drafts and awkwardly placed doorways and windows are unacceptable because they may permit an increase of airborne contaminants. Tissue culture is only feasible when contaminants in the atmosphere are minimized or eliminated.

Tissue culture laboratories should be equipped with suitable electrical power outlets. In areas where power failures are common, a stand by generator is essential for maintaining cultures and conducting work in the laboratory during prolonged power blackouts. Gas outlets for bunsen burners and preparation of medium are necessary. Air and vacuum outlets are desirable and can be economically furnished by a compressed air/pressure/vacuum rotary motor-mounted pump. A ready source of distilled water is important to obtain uniform culture medium consistency. Distilled water from a bottled water company is adequate. Distilled water can be produced in large quantities from tapwater through the use of a demineralizer coupled with a water distillation apparatus, but it is an expensive alternative.

The dishwashing area must be provided with adequate shelf, counter, and sink space. Dishwashing machines may aid in cleaning culture tubes and smaller glassware items; however, large delicate glassware should be hand cleaned. Suitable dishwashing arrangements should be made as the laboratory work begins. To minimize disorder in the laboratory, spacious cabinet and shelf accomodations are necessary to store glassware and chemicals.

Large equipment pieces employed in tissue culture laboratories include: autoclaves, balances, refrigerators, and pH meters. Balances should be of the

top-loading type with a digital readout and have a range of 0.001 to 150 g. Refrigerators are necessary to store the chemicals needed for medium preparation; household types are adequate.

The transfer room should be equipped with a laminar-flow hood that supplies a constantly screened airflow during aseptic transferring. The dimensions of the hood depend on the tissue culture production capacity desired. A dissecting microscope equipped with binocular eyepieces and with a magnification of at least 7X to 42X is beneficial in explant preparations in and out of the hood.

The culture room should be equipped with suitable shelf space and cabinets to incubate cultures. Enclosed cabinets are suitable for dark conditions. Illumination is usually provided, most economically, by using fluorescent lamps of 4- and 8-ft lengths. It is desirable to have at least two illuminations, 100 and 1,000 footcandles (fc). Additional equipment, such as roller drums, shakers, special environmental growth chambers, and roller apparatus, may be used, if necessary.

Staffing the tissue culture laboratory with competent, experienced personnel is invaluable to the success of any project. Suitably trained people with a knowledge of tissue culture and scientific research procedures should be found. Experience in commercial nursery and personnel management is also desirable. Consultation with qualified people in associated fields can prevent costly mistakes or delays in beginning any project.

Preparation of Nutrient Medium

The choice of the original nutrient medium composition will determine the method of date palm vegetative propagation, that is, asexual embryogenesis from callus or through the rooting of lateral buds and shoot tips, and potential division thereafter. Date palm tissues injured as they are excised from parental tissues will brown and die in culture unless an adsorbent is included in the nutrient medium. Activated neutralized charcoal (Sigma Chemical Co., St. Louis, Mo.) added to the medium at 0.3-percent concentration has been found to adequately minimize browning. Callus has been satisfactorily produced from explants on a modified Murashige and Skoog's (*35*) medium containing the ingredients listed in table 3.

Table 3.--*Composition of medium employed to induce callus formation from date palm explants*

Components	Milligrams per liter
Inorganic salts [*Murashige and Skoog* (35)]	
Calcium chloride, $CaCl_2$	322
Cobalt chloride, $CoCl_2 \cdot 6H_2O$	.025
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	.25

Table 3.--*Composition of medium employed to induce callus formation from date palm explants*--Continued

Component	Milligrams per liter
Inorganic salts [*Murashige and Skoog* (35)]--Continued.	
Copper sulfate, $CuSO_4$	0.016
Potassium phosphate, KH_2PO_4	170
Potassium iodide, KI	.83
Potassium nitrate, KNO_3	1900
FeNa EDTA	36.7
Boric acid, H_3BO_3	6.2
Ammonium nitrate, NH_4NO_3	1650
Magnesium sulfate, $MgSO_4$	181
Manganous sulfate, $MnSO_4 \cdot H_2O$	16.9
Zinc sulfate, $ZnSO_4 \cdot H_2O$	8.6
Sodium phosphate, $NaH_2PO_4 \cdot 2H_2O$	170
Carbohydrate source	
Sucrose	30 000
Vitamin sources	
Myo-Inositol	100
Thiamine·HCl	.4
Complex addenda	
Phytagar	8000
Charcoal, activated, neutralized (Sigma)	3000
Phytohormones	
2,4-dichlorophenoxyacetic acid (2,4-D)	100
N-(Δ^2-isopentyl) adenine (2iP)	3

Each component is added separately and dissolved when the medium is prepared from pure chemicals. Premeasured, mixed, and pH adjusted plant tissue culture medium formulations are also available from various commercial laboratories. These media may be used with a minimum of laboratory experience and time. Amateur and commercial operators may wish to investigate this alternative method of medium preparation to minimize expense. Phytohormones and other additives not normally included in the prepared medium may be added separately. The pH of all nutrient media should be set at 5.7 ± 0.1 with 0.1 N HCL or NaOH before the addition of agar. Adjustment of pH in a preadjusted medium is necessary after any additional components are added. Agar is added last, after the adjustment to the final volume.

For uniform solidification, the medium must be heated and stirred to dissolve the agar. Agar is dissolved in a container--which is at least twice the volume of the medium--on a stirring hotplate gas burner with constant agitation or by autoclaving for 5 minutes. After the agar has been dissolved, the medium is distributed into culture vessels. Nutrient medium may be dispended in uniform aliquots through several methods such as repeating pipettors, dispensing burets (for example, 250, 500 or 1000 ml) with Teflon stopcocks, a large funnel fitted with plants tubing and a pinch clamp, or an automatic pipettor.

Probably, the most accurate and rapid method to dispense large volumes would be with an automatic pipettor. Medium is dispensed into 25- by 150-mm Bellco culture tubes at the rate of 25 ml per tube. The tubes are capped with Bellco plastic kaputs or closures. When tubes are to be illuminated, the use of transparent kaputs is advised as the closures of choice. Nutrient medium is sterilized by autoclaving for 15 min. Tubes may be cooled upright or slanted at a 45° angle in their stainless steel racks to provide an increased planting surface.

To root lateral buds and shoot tips, a nutrient culture medium, containing either 0.1 or 1.0 mg/L NAA or 2,4-D, is used. Low concentrations of auxins with charcoal will allow explants to enlarge and to develop leaves and roots without unwanted callus production.

Nutrient medium should be used within 72 hr of preparation to ensure satisfactory activity of its components.

Explants

Selection

Correct selection of original explants for tissue culturing is one of the most important steps to successful laboratory propagation. To undergo laboratory morphogenesis *in vitro*, tissue culture explants must be meristematic and healthy. Therefore, young, undifferentiated explants should be sought from healthy vigorous trees. Such explants may be produced from adult trees and from offshoots. Only the aerial third of the tree will contain tissue suitable to tissue culture. Offshoots are the best source of all explant materials. Furthermore, increased fruit yields from the denuded adult tree are obtained once offshoots have been removed (*65*).

Shoot tips and leafy lateral buds are highly meristematic tissues. Most lateral buds from adult trees are unusable because they are involved exclusively in fruit production. Adult trees are a good source of inflorescence buds. Offshoots are dissected through the acropetal removal of the leaves with the aid of a hatchet and a serrated knife. Lateral leafy and inflorescence buds are found at the base of the leaf axils (fig. 3). Excised lateral buds often vary in size and development within the same offshoots.

Examination of dissected offshoots, containing 20 to 40 leaves, reveals that only a few buds are meristematically active (table 4). These meristematic buds are precursors of future offshoots and are characterized as having early leaf development. Generally, a single offshoot may have only two or three meriste-

Table 4.--*Incidence of average numbers of culturable buds (>3 mm in length) and appendages from offshoots of date palm varieties and hybrids*[1]

Variety	Buds per offshoot (average)					
	Total	Decomposed buds	Vegetative appendages	Reproductive appendages	Buds with leaves	Buds without leaves
Barhee	11.6	2.8	1	0.2	2.6	5
Deglet Noor	14.6	4.2	1	2.8	2.6	3.6
Express seedlings:[2]						
Male	20.4	2.4	1.3	1.5	1.2	14
Female	20	2.6	1.3	2.2	2.6	11.4
Halawy	11.6	3.4	1.4	1.6	.6	4.6
Khadrawy	14	3.4	3.2	1.8	1.8	3.8
Medjool	15.6	3	2.6	1.8	2.7	5.5
Thoory	10.6	7.8	1.4	.8	2	7.6
Zahidi	12	4.8	1	2.2	.8	3.2

[1]Offshoots were obtained from 7-year-old clonal palms; weight of offshoots varied from 3.6 to 10.4 kg; 5 offshoots were used to represent each variety.

[2]Offshoots were obtained from 7-year-old seedling date palms in which Empress is the female parent; 6 female trees with 19 offshoots and 5 male trees with 22 offshoots were used.

matically active buds. Suitable leafy buds measure 8 to 20 mm in length and 4 to 10 mm in width (fig. 4). Buds without leaf differentiation failed to develop *in vitro*. Immature inflorescence buds and small spadices may be used as the source of rachilla tissue and attached flower buds.

Only one apical tip may be obtained from each shoot. Tips are dissected and trimmed to an explant size of 5 to 10 mm in length and 4 to 10 mm in width.

Meristem tissues capable of undergoing morphogenesis *in vitro* may be obtained from young stem tissue located directly beneath the shoot tip. Older meristem tissue, at a greater physical distance from the shoot tip, is physiologically less capable of undergoing differentiation in culture. Meristem explant tissue is removed from the uppermost 3 cm of the shoot tip region by using a No. 2 cork borer. Meristem tissue may be procured from both adult and juvenile plants.

Excised date embryos may be obtained from either ripening or mature fruit. Petiole and rachis tissues obtained from immature organs, and root tips and young growing tips from germinating date seeds have also been planted *in vitro*.

Planting on Nutrient Medium

Disinfection of tissues is necessary to eradicate surface contaminants. Any contaminant will interfere with the growth of explants *in vitro*. Original explants are cut into suitable sizes, wrapped with two layers of cheese cloth, and submerged in a chemical sterilant in a culture vessel, such as a 25- by 150-mm or 38- by 200-mm culture tube. The vessel should be tapped or shaken periodically to dislodge air bubbles that may develop on the surface of the explants.

Date palm explants are extremely difficult to surface sterilize. Normally, most plant tissues are chemically sterilized by a 10:1 or 20:1 sodium hypochlorite (bleach) solution. Excised date tissues require surface sterilization by immersion in 5:1 bleach solution containing one drop of Tween-20 (polyoxyethlene sorbitan monolaurate) per 100 ml solution for 15 min and then rinsing three times with sterile water. A bleach solution should be used immediately after it is prepared because it breaks down photochemically in light. Following chemical sterilization, tissues should be planted immediately to prevent browning and dehydration.

Explants removed from the tissues of origin are immediately transferred and soaked in cold antioxidant solution of 150 mg/L citric acid and 100 mg/L ascorbic acid to retard browning. Explants may be kept overnight in the refrigerator at 6°C in this solution if necessary without any harmful effects on subsequent growth when planted. The use of a long-necked, 7-inch serrated forceps and a surgical scalpel equipped with a No. 11 blade will aid in the further trimming of explants. All subsequent operations should be conducted in a laminar flow hood, using aseptic techniques.

Shoot tips and lateral buds are trimmed to desired sizes and chemically sterilized, and an additional leaf or two are removed aseptically. Meristem explants are cut into 2- to 3-mm-thick sections after sterilizing. Following

aseptic preparations of tissues, a final dip in bleach solution for 5 sec without rinsing is administered to explants before planting on nutrient medium. Mature seeds may be soaked in tapwater for 48 hr to remove excess fruit pulp and hydrate the dry seed to facilitate embryo excision before surface sterilization. During soaking, the water should be changed frequently. Seeds are surface sterilized in a 50-percent bleach solution then split open lengthwise, using an anvil hand cutter. The exposed embryo is removed and planted with a scalpel.

Occasionally, multiple embryos occur within the same seed. The origin of these additional embryos is not known. Inflorescence buds and maturing spadices may be surface sterilized intact and should be cut open under aseptic conditions. Internal rachilla and attached flower buds may be cut into sections 1 to 2 cm long and planted on the surface of the medium without further chemical sterilization.

Cultural Conditions

The cultural conditions to which the explants are exposed influence the type of morphogenesis obtained *in vitro*. To produce rooted buds and tips, explants should be cultured in a temperature-controlled room at 27°C under a 16-hr daily photoperiod exposed to a 100-fc grow light. Callus production from explants should be initiated by incubation in complete darkness. Later, the transfer of callus cultures to a medium, devoid of hormones, in light will enhance plantlet development. After the first few leaves are formed, cultured plantlets may then be transferred to a higher light intensity, such as a 1,000-fc grow light.

Inexpensive light meters may be used to monitor illumination, in footcandles, during incubation to maintain uniformity of light intensity. Defective lights should be replaced immediately since they may be safety hazards and may emit irregular illumination intensities.

According to studies performed at this location, date palm seeds germinate and grow well from 25° to 30°C; although, 35°C has not been found to be harmful to their development.

Contamination of cultures may arise from poor aseptic technique, improper air circulation, or unsanitary laboratory conditions. Culture tube closures are vented slightly to permit some aeration during incubation. This closure venting may also contribute to medium dehydration and contamination. In such an event, the tubes may be sealed temporarily with parafilm until the causal factor is corrected.

Growth of Cultures and Reculturing Procedures

All tissue cultures must be recultured to fresh nutrient medium at regular intervals to replenish exhausted nutrients and allow further morphogenesis. Reculture to fresh medium every 8 weeks is recommended.

Callus Production and Plantlet Generation

Plantlets may be generated from excised embryos, immature inflorescences, lateral buds, mersitem, and shoot tip explants. Callus production is faster and more prolific from lateral buds and shoot tips because they are more highly meristematic than the other explant tissues. Embryogenetic callus is usally produced from excised embryos, lateral buds, and shoot tips after one to two recultures in medium containing 100 mg/L 2,4-D. This callus readily proliferates and may be subcultured. Inflorescence and meristem rarely produced callus and then only after several recultures. Usually, callus produced from these sources is not embryogenetic.

Callus gives rise to plantlets through an asexual embryogenesis process. This process is not synchronous within the callus mass; asexual embryos and plantlets at various stages of development coexist within a single culture (fig. 5). Plantlets produced from callus cultures should be removed and planted separately on fresh medium to foster their subsequent development. Residual callus and younger embryoids may be recultured to produce more plantlets. The growth rate of callus from palm explants is related to the type and concentrations of auxin used in the culture medium (table 5). Subculturing callus and the separa-

Table 5.--*Influence of growth regulators on the growth of lateral buds and shoot tips from date palm seedling offshoots*

Plant growth regulator in medium		Weight of culture per treatment	Callus per treatment	Length of culture per treatment
	Mg/L	*Mg*	*Percent*	*Cm*
2,4-D	0.0	4.00 ± 0.95	0	1.3 0.7
	.1	2.50 ± .12	40	1.4 .3
	1.0	1.36 ± .14	60	1.9 1.0
	10.0	.92 ± .76	70	1.2 .2
	100.0	.32 ± .11	80	1.0 .4
NAA	.1	1.88 ± .82	20	1.5 .3
	1.0	1.35 ± .37	20	1.2 .1
	10.0	1.48 ± .97	30	1.2 .1
	100.0	.54 ± .21	50	1.0 0.1

tion and culturing of plantlets is a time-consuming labor-extensive process. Refinement of this procedure will determine the efficiency of the *in vitro* propagation method for palms.

Transplanting to Free-Living Conditions

Plantlets about 12 cm in length with distinct taproots and two or three early foliar leaves are capable of surviving transfer to free-living conditions. The initial size of the plantlets is a critical factor in their survival in soil conditions (table 6). With the aid of long forceps, plantlets were removed from

Table 6.--*Effect of plantlet size on their survival under free-living conditions*[1]

Height of plantlet	Survival	
	4 weeks	8 weeks
Centimeters	*Percent*	*Percent*
10-12	100	100
8-10	100	70
6-8	50	30
4-6	40	10
2-4	30	0
1-2	0	0

[1]Plantlets cultured on a peat-vermiculite mix under plastic tents in a shaded greenhouse.

the nutrient medium and soaked in distilled water for 30 min to avoid dehydration and to remove excess medium. Individual plantlets are separated with care from the callus mass and other plantlets to avoid root and shoot damage, are rinsed three times with distilled water, and are allowed to soak an additional 30 min. A 0.5-percent benomyl spray treatment is administered to each plantlet before transplanting to soil medium.

Plantlets were transplanted into a mix of equal portions of peat moss and vermiculite soil mix in 7.6-cm-diameter Jiffy pots covered with plastic lids (fig. 6). Plantlets were incubated in a growth chamber for 2 weeks at 30°C under 800 fc at a 16-hr photoperiod and were watered every other day. For their first 2 months of development, plantlets were sprayed with benomyl and watered with one-fourth strength Hoagland's solution once a week.

After 4 weeks, plantlets were placed in a shaded greenhouse where they remained for the next 2 to 3 months. Thereafter, plants grew in normal greenhouse conditions. Average temperatures in the greenhouse were from 25° to 30°C. Gradual acclimation of the plantlets to greenhouse humidity was achieved over several months by puncturing the pot cover. Free-living plantlets have been produced that are several months old (figs. 6 and 7).

Supplementary Notes on Date Palm Tissue Culture Responses

Mode of Asexual Embryogenesis *In Vitro*

The orgin of asexual embryos from date palm callus has been described (*69*, *71*). Callus initiated and maintained on nutrient medium with 2,4-D was found to be heterogenous--composed of disorganized vacuolated cellular groups and compact aggregates (figs. 8 to 14). The vacuolated cells were not involved in the morphogenetic processes and were presumed to be sloughed-off tissues. The compact aggregates were composed of various cellular types, including meristematic loci and cells, and semivacuolated parenchymalike cells. These meristematic loci were the proembryonic precursors, and they were thought to be derived from single dividing cells (figs. 12 and 13). Dividing meristematic cells were found on the periphery and the interior parts of the aggregate clump.

Single cells divide transversely to produced two-celled, spherical proembryos (fig. 12). Subsequent divisions resulted in formation of 8- and 16-celled spherical embryos enclosed by a thick cell wall (fig. 13 and 14). This enclosing cell wall, ruptured by further divisions, formed a multicelled bipolar embryo, consisting of meristematic and vacuolated ends, the shoot and root pole region, and the haustorium of the cotyledon precursors (fig. 15). This bipolar embryo elongated and differentiated further to produce and embryo that resembeled the embryo stage normally found in a ripe seed before germination. Transfer to medium devoid of hormones allowed this bipolar structure to germinate and to produce a plantlet with distinct root and shoot growing tips. Within 2 weeks on medium without 2,4-D, the bipolar embryos developed at well-defined root and shoot poles.

On medium without 2,4-D, the consistency of the callus changed, becoming looser and composed of enlarging embryos in various stages of development (fig. 16). The haustorium end of the bipolar embryo protruded from the callus mass while the root and shoot pole ends remained embedded (fig. 17). The root pole region remained in physical contact with the surrounding callus until the expansion of the plumule and primary root and was actively meristematic and differentiating throughout this period.

In contrast, the haustorium was meristematically inactive and much reduced in size compared with that found in the seed. The haustorium had a vestigial nutritive role during the asexual embryogenesis process; apparently, nutrients were absorbed directly by the growing and developing root and shoot region embedded within the callus mass. Subsequent embryo stages of development and germination corresponded closely to zygotic seed and excised embryo events (figs. 18, 19, and 20).

Other Morphogenetic Responses

Rachillae with attached flower buds from maturing inflorescences, cut a few weeks before their opening, can be induced to produce a friable callus after several recultures on medium with high concentrations of 2,4-D. This callus may be subcultured and may give rise to roots (fig. 21); however, these roots have

not produced corresponding shooting structures. Interestingly, when culturing male flower buds, vestigial carpels enlarged and became prominent (fig. 22). Carpels from cultured female buds also enlarged. Only immature inflorecence tissues, obtained from buds several months before their visable outgrowth, were capable of producing an embryogenetic callus.

Little success has been achieved with production of callus from petiole, rachis tissues, and root tips. Root tips may be induced to enlarge and produce lateral roots, but callus production has not been obtained. Excised embryos from seeds cultured on medium devoid of hormones in light will produce seedlings (fig. 19). Cocultured excised zygotic and additional embryos produce plantlets equally well.

Rooting Buds and Shoot Tips

On nutrient medium containing low levels of auxins, such as 0.0, 0.1, and 1.0 mg/L 2,4-D or NAA, shoot development from buds and shoot tips was enhanced (figs. 23 and 24). Within a few weeks, cultured tips and buds produced leaves. These leaves turned green in light and enlarged considerably. After several recultures, these cultures also may produce roots, which results in the production of plantlets (fig. 25). Root initiation was infrequent, and its mechanism has not been determined.

Quality Control Tests

Propagation of a substantial number of palm species has been proposed by various authors using tissue culture techniques (*4*, *67*). Genetic variation in higher plants regenerated from cell and tissue cultures has been reported (*8*, *25*, *34*). The occurrence of mutants or sports is always a possibility. Palms are long-lived plants that do not flower and fruit until 3 to 7 years following the planting of seed or offshoots. Though tissue-culture propagation techniques may increase the number of palms produced per tree, no method is known to accelerate the growth rate beyond the normal time required for development. Therefore, evaluation of tissue cultured palms for their clonal status, based on comparison of vegetative and reproductive characteristics, must wait for years. This section reports preliminary studies undertaken to verify the clonal nature of palms produced through tissue culture. Morphological and enzymatic analyses were conducted on plantlets derived from tissue culture to determine their clonal status.

Callus-derived plantlets were produced through asexual embryogenetic process that corresponded to the *in vitro* developmental sequence (fig. 26). Morphological differences between tissue cultured and seedling palms occurred frequently. Seedling palms are initially more vigorous than plantlets in soil conditions. Seedling palms have an erect sharp-pointed first foliar leaf (plumule) that usually extends to more than 15 cm in length. The initial leaves of the tissue cultured palms were 5 to 10 cm in length and tended to bend and curl; no sharp-pointed plumule was observed (fig. 17). This may be related to the absence of a nutritional endosperm for plantlets.

Unusual callus lines and plantlets may be due to mutation or sports and should be discarded when detected. Healthy callus is composed of yellow-white nodules. Unhealthy callus is usually brown with poor plantlet-producing capacity and a low growth rate.

The general procedures used for starch gel electrophoresis and gel staining have been described previously (72, 74, 75). The genetic control of isozymes of alcohol dehydrogenase (Adh), esterase (Est-1, Est-2), glutamate oxaloacetate transaminase (Got-1, Got-2), phosphoglucose isomerase (Pgi-1), and phosphoglucose mutase (Pgm-1) has been examined in date palms (72, 75). The segregation ratios based on parents and progeny for seven enzyme systems followed a predictably Mendelian inheritance pattern. All cultivars derived from offshoots should show their distinct gene-enzyme patterns when they are compared with other cultivars.

Enzymatic comparisons of the seven gene-enzyme systems for clonal and tissue culture progency leaves are shown in table 7. There was no difference for any of the isozymes tested to date. Apparently, there was no difference in intensity nor location of bands for these enzyme systems.

Table 7.--*Comparison of leaf genotypes for callus-derived plantlets*[1] *and parental trees*

Tissue source	Genotypes						
	Adh	Got-1	Got-2	Est-1	Est-2	Pgm-1	Pgi-1
Medjool plantlet.	FF	FF	FF	SS	NN	FS	FS
Medjool adult.	FF	FF	FF	SS	NN	FS	FS
Seedling plantlets.	FF	FF	FF	SS	AA	FF	FS
Seedling parent.	FF	FF	FF	SS	AA	FF	FS

[1]Plantlet tissue taken from 6-month-old leaf tissue; 5 replicate-samples employed in each analysis.

The isozyme test provides for some degree of confidence that the tissue cultured palms are clonal; however, few gene-enzyme systems in the plant were analyzed. Changes in gene-enzyme systems not yet determined may occur; furthermore, most metabolic differences might not affect morphological characteristics (*24*).

Cryostorage of Date Palm Tissue

Cryostorage of plant tissues offers a means to conserve rare or infrequently used fruit tree germplasm collections, such as that of the date palm, and requires

minimal maintenance and expense for potentially indefinite time periods. Low temperature storage suppresses cell division and DNA synthesis for prolonged periods. Immediate and direct applications of frozen tissue banks are possible. Date and other palm field collections, which face liquidation through mounting economic and spacial pressures, may be preserved through this new technology.

Embryogenetic date palm callus was subjected to cryogenetic treatments. After storage for several months, the callus revived and produced plantlets. The plantlets were transfered to the soil.

Date palm callus was soaked in a cold, half-strength cryoprotective mixture (PGD), consisting of polyethylene glycerol (Carbowax 6,000, 10 percent weight per volume (w/v)), glucose (8 percent w/v) and dimethylsulfoxide (10 percent w/v) for 5 to 10 min in a petri dish on ice. The concentration of PGD was increased by the addition of two or three times the original volume with full strength PGD while mixing gently for 5 min. This callus was centrifuged, and 1 ml full strength PGD was added.

Callus was treated in a programmable freezer. The freezer was adjusted to 4°C and programed to reduce the temperature at 3° to -4° and then to 1° per min after ice crystallization was initiated by a programed brief application of liquid nitrogen. When samples reached -30° (after 26 min) they were transferred to liquid nitrogen (-196°). Callus was subjected for 3 months to liquid nitrogen temperatures and then thawed rapidly by swirling tubes in a 60° water bath until the ice around the tubes melted. The PGD cryoprotective mixture was diluted by gradual additions of 3 percent sucrose to minimize osmotic shock.

Cultures were transferred to agar nutirent medium without hormones and incubated. Thawed callus produced callus and plantlets following a quiescent period of 4 to 8 weeks. Further long term cryogenetic tests are ongoing with date palm callus.

CONCLUSIONS

A practical method of palm tissue culture was achieved by production of plantlets from callus. Plantlet generation from callus is not a synchronous process. Only a few plantlets per tube were of sufficient size to be transferred to free-living conditions.

Apparently, some type of competition among plantlets occurs within the culture vessel, preventing numerous large plantlets from developing. Continuous subculturing, which included separation and transplanting plantlets and callus to fresh medium, was required to produce a substantial number of plantlets *in vitro*. The palm tissue culture propagation method is labor intensive. Palm progagation difficulties are compounded by their naturally slow growth rates as compared with other plant tissues (*28*, *61*).

Once plantlets are produced, their rate of growth cannot be expected to exceed normal development. The clonal nature of plantlets produced from callus must be established. Only through repeated, large-scale propagation and subsequent evaluation tests of the progeny can palm plantlet clonal status be determined.

Three research directions to develop a practical method to rapidly propagate palms are now available: (1) development of a quality control test to determine clonal status of plantlets from tissue culture, (2) development of a method to mass produce palms *in vitro* and to minimize the labor required, and (3) development of a suitable method to root and to accelerate division of lateral buds and shoot tips.

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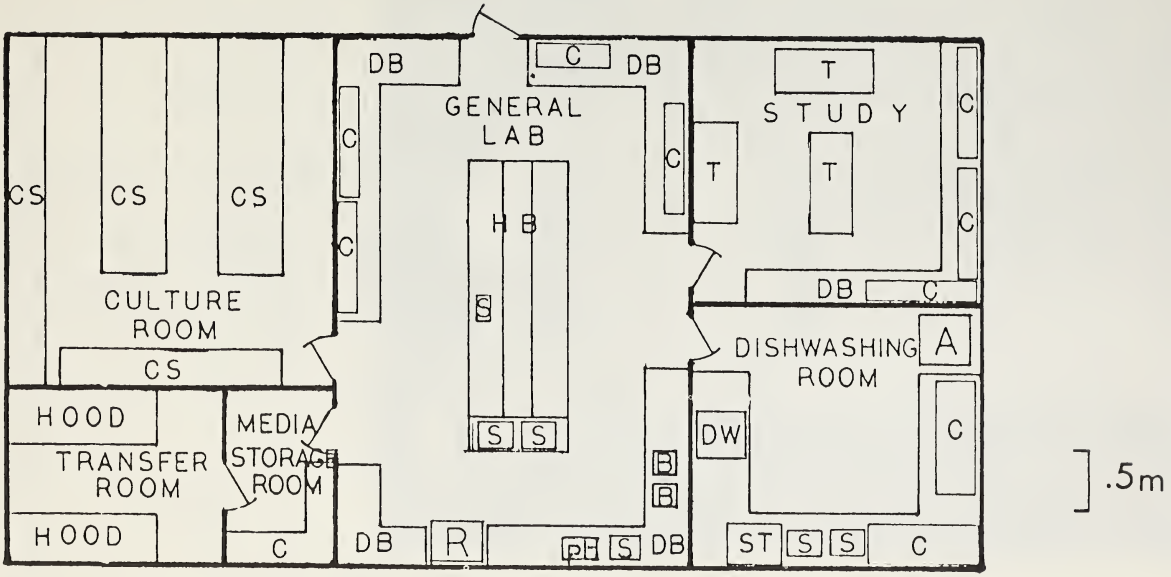


Figure 1.--Diagrammatic representation of a small commercial tissue culture laboratory. *A*, autoclave; *B*, balance; *C*, cabinet; *CS*, culture shelves; *DB*, desk-bench; *DW*, dishwasher; *HB*, high bench; *pH*, pH meter; *S*, sink; *T*, table.

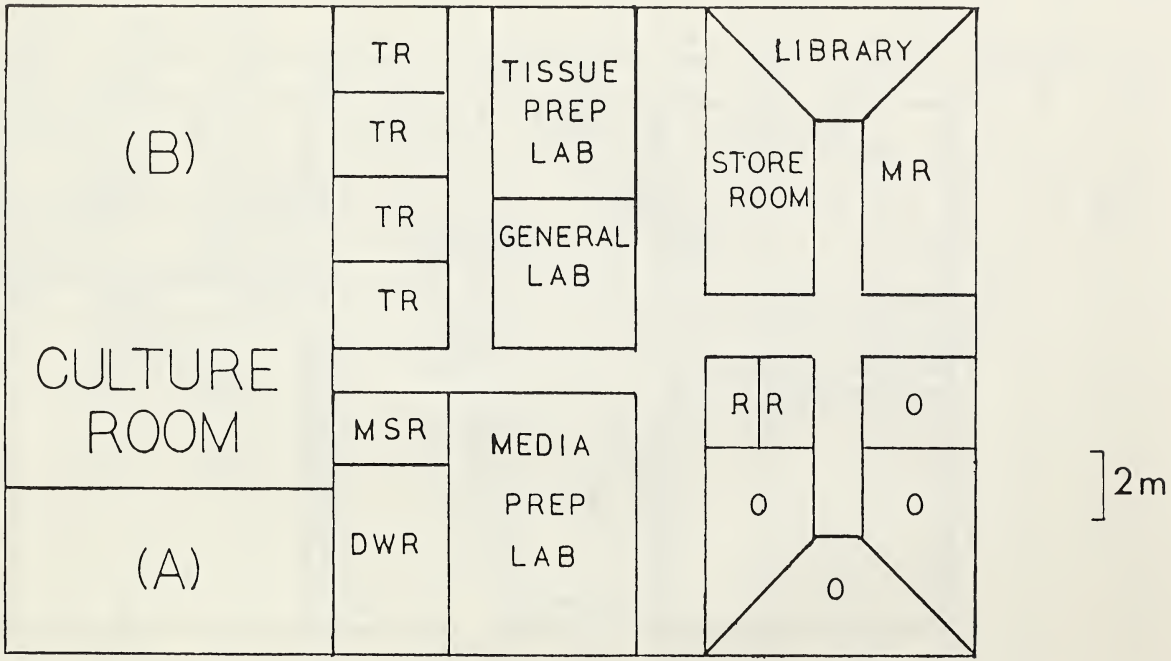


Figure 2.--Diagrammatic representation of an idealized large-scale commercial tissue culture complex. Note that only one entrance exists and that as one proceedes into the complex the sanitation standards increase. Culture room (*A*), devoted to use of incubators, roller apparatus and drums, and shakers; Culture room (*B*), devoted to culture shelves only; *DWR*, dishwashing room; *MSR*, media storage room, *MR*, meeting room; *O*, offices; *RR*, restrooms; *TR*, transfer room.

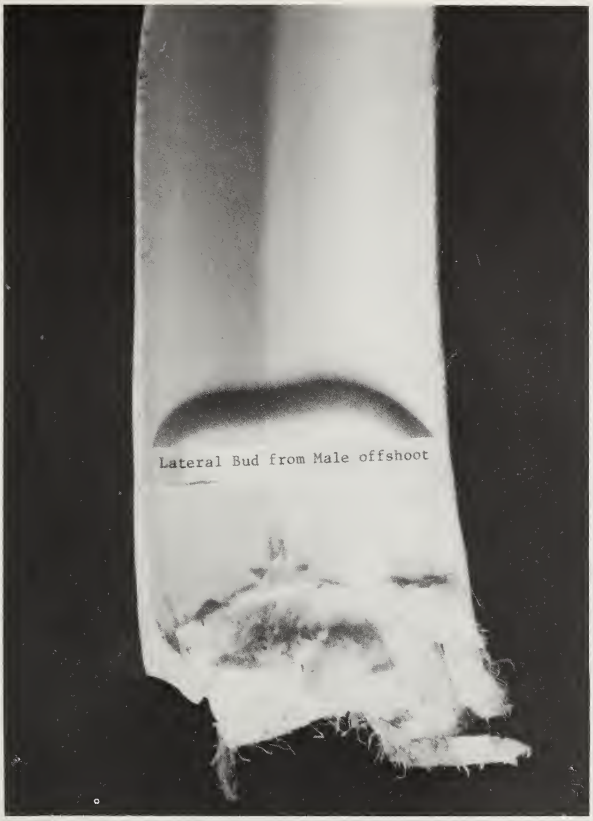


Figure 3.--Leaf petiole base from a dissected offshoot. Note bud at base of leaf. In this instance, an undifferentiated bud is present (about 1/4 X).

Figure 4.--Examples of lateral buds and shoot tips obtained from date palm offshoots. The shoot tip is the explant on the far left; the other three explants are various types of lateral buds that may be cultured *in vitro* to initiate plantlets through rooting or callus production (about 3 X).

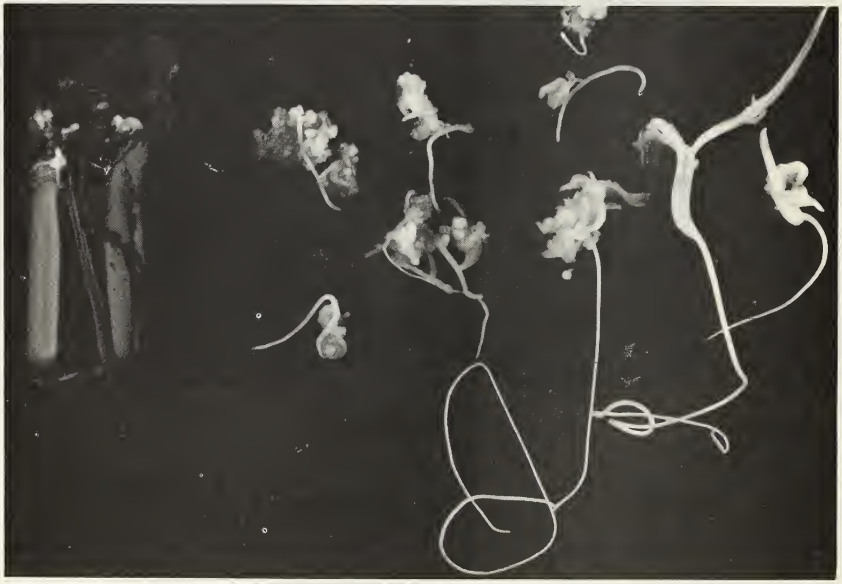
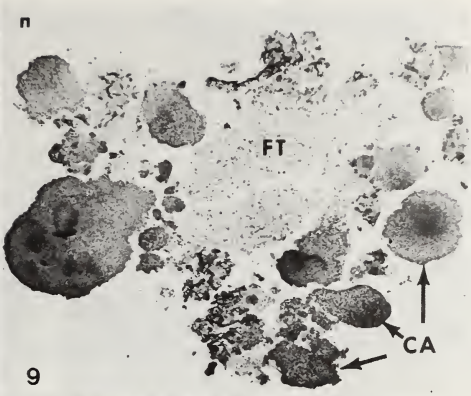
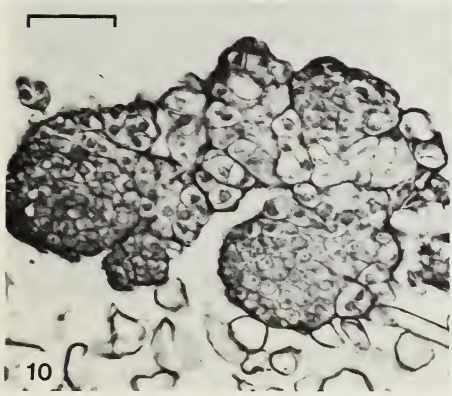


Figure 5.--Example of the nonsynchronous growth condition exhibited by a single date palm callus culture. Note that only a few larger plantlets and embryos are produced per culture (about 1 X).

Figure 6.--Free-living plantlets. *Left*, Date palm enclosed by a transparent plastic cover to retain high humidity; and *right*, plantlet with cover removed. Plantlets are about 16 to 20 weeks old (about 1/2 X).

Figure 7.--Sequential transfer of plantlets from *in vitro* to soil conditions. Plants are transplanted to larger pots to accommodate their increased growth in the greenhouse (about 1/5 X).





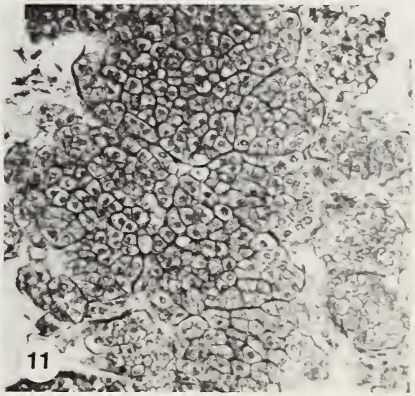


Figure 8.--Example of date palm callus produced on a nutrient medium containing 100 mg/L 2,4-D and 3 mg/L 2iP. Note that callus consists of nodular bodies and a matrix of friable cellular tissue. These nodular bodies are embryogenetic compact aggregates (about 200 X).

Figure 9.--Transverse section through date palm callus culture, 2 weeks after culture. Several compact aggregates (*CA*) are dispersed among a matrix of loose friable tissue (*FT*). (Scale line=50 µm.)

Figure 10.--An example of embryo budding from a compact aggregate. (Scale line= 50 µm.)

Figure 11.--Occurrence of meristematic loci within a callus aggregate. Note the thick wall fragmentation lines demarcating loci. (Scale line=50 µm.)

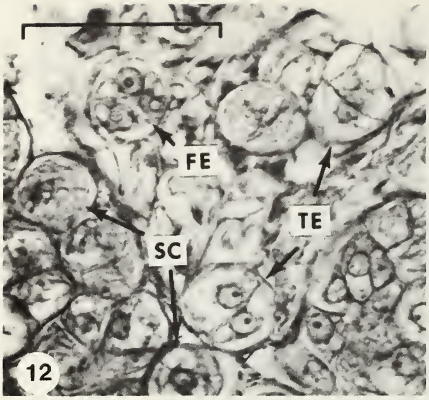


Figure 12.--Section through date palm callus. Transverse division of the single cell (*SC*) embryogenetic progenitor yields a two-celled embryo (*TE*). A further division yields a four-celled spherical tetrad embryo (*FE*). (Scale line=50 µm.)

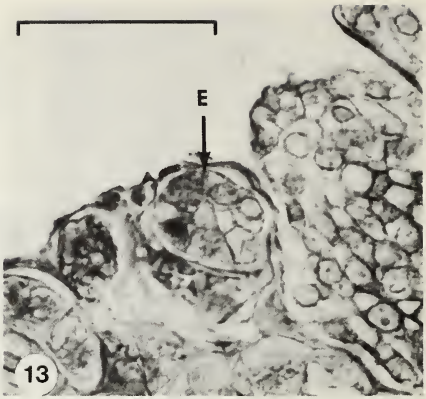


Figure 13.--Example of an eight-celled proembryo. Note the thick exinelike wall surrounding the proembryonic structure. (Scale line=50 µm).

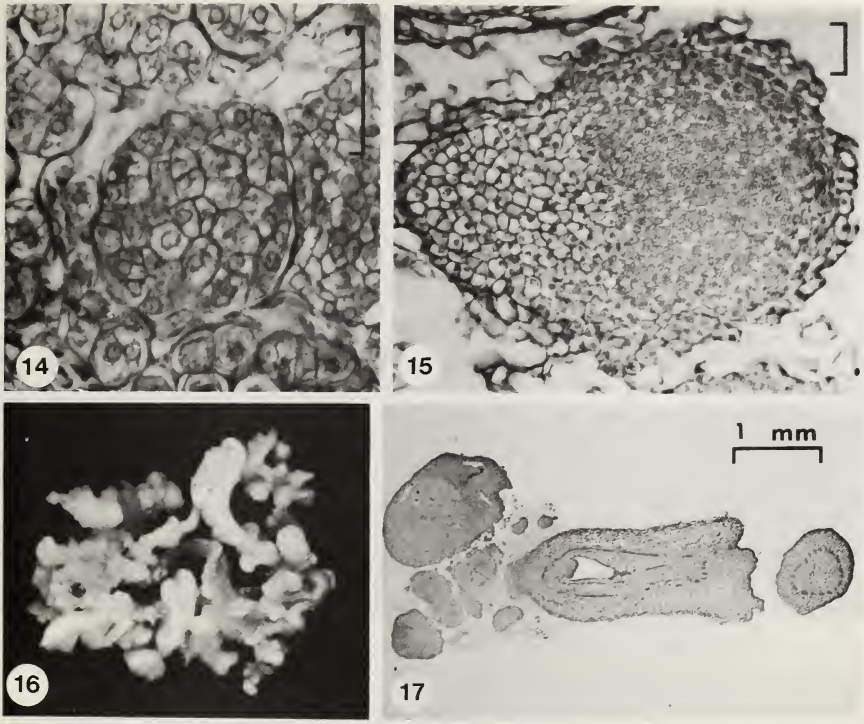


Figure 14.--Multicellular spherical proembryonic structure on medium containing 2,4-D and 2iP. (Scale line=50 µm.)

Figure 15.--An example of an early bipolar proembryonic structure prior to epicotyl and haustorium organization in medium containing 2,4-D and 2iP. (Scale line=50 µm.)

Figure 16.--An example of date palm callus culture on nutrient medium devoid of 2,4-D and 2iP, 2 weeks after culture. Note enlarged embryonic structures (about 200 X).

Figure 17.--Orientation of the asexual embryo within the callus on medium devoid of 2,4-D and 2iP, 2 weeks after culture. Embryoids root pole region is embedded within the callus of origin. (Scale line=1 mm.)

Figure 18.--Germination of a palm seed. From left to right: protrusion of cotyledon through centrally located pore, 2 weeks old; elongation of cotyledon and developing primary root, 4 weeks old; emergence of first leaf through slit-shaped opening of cotyledonary sheath, 6 weeks old; free-living seedling, 10 weeks old (about 1/2 X).

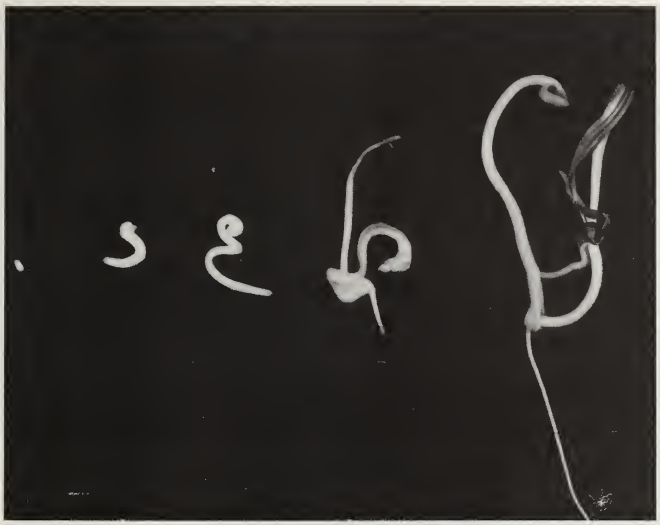


Figure 19.--Germination of an excised embryo. From left to right: freshly excised embryo from mature seed; early cotyledonary sheath elongation stage, 2 weeks in culture; later cotyledonary sheath elongation stage, 4 weeks in culture; emergence of the primary root and the plumule from the cotyledonary sheath, 6 weeks in culture; plantlet with photosynthetic leaves, 8 weeks in culture (about 1 X).

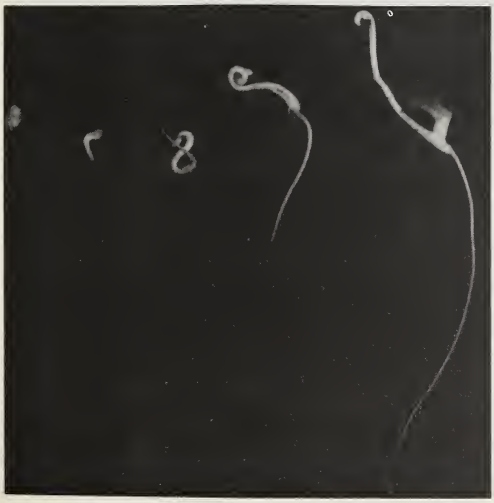


Figure 20.--Sequence of germination of asexual embryos from callus on nutrient medium devoid of 2,4-D and 2iP. From left to right: cotyledon of asexual embryo protrudes from the callus aggregate, 2 weeks in culture; excised embryoid at early cotyledonary elongation stage, 4 weeks in culture; later cotyledonary elongation stage, 6 weeks in culture; emergence of first leaf and elongation primary root, 8 weeks in culture (about 1 X).

Figure 21.--Example of morphogenetic responses obtained from culturing flower bud and rachilla cultures on nutrient medium containing 2,4-D and 2iP, after 8 weeks in culture. Note the development of rootlets and friable callus (about 2 X).

Figure 22.--Examples of psuedocarpels developed from cultured male flower buds and attached rachilla after 8 weeks in culture (about 2 X).

Figure 23.--Shoot tips and lateral bud cultures with enlarging leaves 4 weeks in culture on nutrient medium with 0.3 percent activated charcoal (about 2 X).

Figure 24.--Lateral bud and shoot tip cultures with enlarging leaves after 3 months in culture on medium (about 2 X).

Figure 25.--Examples of rooting of lateral buds after 4 months in culture. Roots are of adventitious origin and occur infrequently (about 4 X).

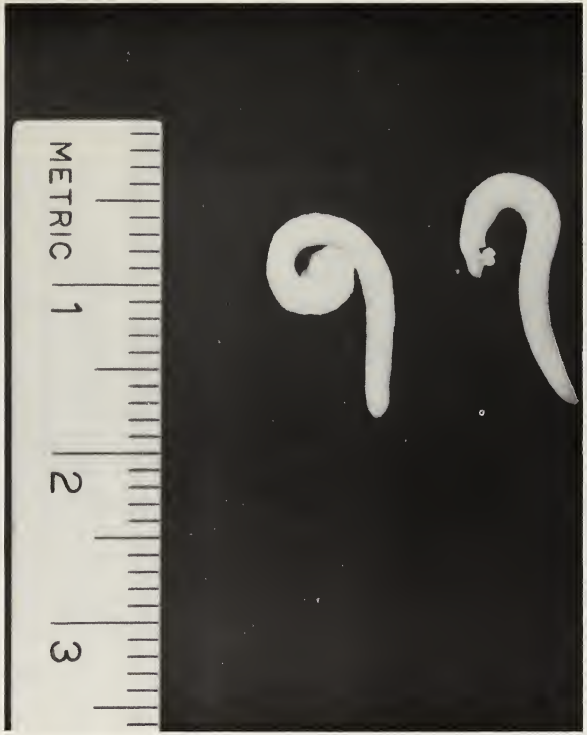


Figure 26.--Comparison of excised zygotic (left) and asexual embryos (right) at the embryo elongation stage of development. Note that the suctorial haustorium is vestigial and much reduced in size (about 3 X).

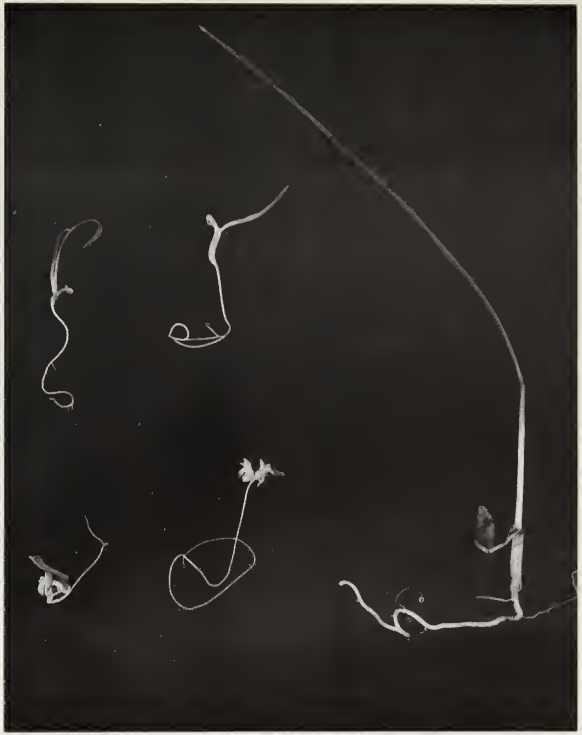


Figure 27.--Comparison of 12-week-old plants produced from tissue culture (right) and a single germinating date palm seedling (left). Note the variation on plantlet when produced from calli. Foliar leaves are much reduced in size compared with normal seedling of same age (about 3 X).

Chemicals Mentioned in This Publication

Common Name	*Chemical Name*
adenine	6-aminopurine.
benomyl	[1-[(butylamino)carbonyl]-1*H*-benzimidazol-2-yl] carbamic acid methyl ester.
citric acid	2-hydroxy-1,2,3-propanetricarboxylic acid.
chloramine-T	*N*-chloro-4-methylbenzenesulfonamide sodium salt.
dihydroxynaphthalene	same.
dimethylsulfoxide (DMSO)	sulfinyl bis [methane].
glucose	*D*-glucose; dextrose.
glutamine	2-aminoglutaramic acid.
gibberellic acid (GA_3)	2β, 4aα, 7-trihydroxy-1-methyl-8-*H*bβ-gibb-3-ene-1a, 10β-dicarboxylic acid, 1, 4a-lactone.
8-hydroxyquinoline 8-hydroxyginoline.	8-quinolinol; phenopyridine.
indoleacetic acid (IAA)	indole-3-acetic acid.
indolebutyric acid (IBA)	3-indolebutyric acid; 4-(3-indoly) butyric acid.
kinetin	*N*-(2-furanylmethyl)-1*H*-purine-6-amine.
mercury chloride	mercurous chloride; calomel.
napthaleneacetic acid (NAA)	α-naphthaleneacetic acid.
peracetic acid	ethaneperoxoic acid; acetyl hydroperoxide.
PGD	polyethylene glycerol: glucose: dimethylsulfoxide.
poleyethylene glycerol	α-hydro-ω-hydroxylpoly(oxy-1,2-ethanediyl).
polyvinylpyrrolididone	same.
sucrose	β-D-fructofuranosyl-α-*D*-glucopyranoside.
2IP	N^6-(Δ2-isopentyl)adenine.
2,4-D	2,4-dichlorophenoxyacetic acid.
Tween-20	polyoxyethylene sorbitan monolaurate.

Adsorbents. Substances that take in and retain detrimental compounds released by the explant, thus minimizing their adverse effect on subsequent tissue growth in culture.

Adventitious. A structure arising from an unusual place, for example, buds produced at places other than from leaf axils and roots growing from stem or leaves.

Agar. A solidifying agent used in nutrient media preparations and obtained from certain types of red algae (Rhodophyta).

Alleles. Alternate forms of a gene at a particular locus on a chromosome.

Aseptic. A sanitary and sterile situation.

Asexual embryogenesis. The sequential series of events whereby an embryo arises from somatic tissue and analagous to the development of a germinating sexual embryo.

Auxins. Natural or artificial types of phytohormones characterized as substances that promote cell elongation (that is, IAA, IBA, NAA, p-CPA, 2,4-D). In tissue culture, these substances are often associated with callus and root production.

Axillary bud. A bud found at the axil of a leaf (synonymous with lateral bud).

Callus. Dividing parenchymalike cellular tissue devoid of macroscopic organized structures.

Carbohydrates. Organic compounds composed of carbon, hydrogen, and oxygen; for example, sugars and starchs.

Cell. Structural unit of living organisms; usually a plant cell consists of protoplasm surrounded by a cell wall in plants.

Chromosome. Nucleoprotein body containing genes. The number is constant amoung species, and the chromosomes become visible and conspicuous during mitosis and meiosis.

Cotyledon. The initial leaf of the embryo.

Complex addenda. Chemically undefined compounds added to nutrient media to stimulate growth.

Cryobiology. Study of low-temperature storage of organisms and their tissues or organs.

Culture. The living tissue grown *in vitro* within a single culture vessel.

Cytokinins. Natural or artificial types of phytohormones characterized as substances that promote cell division (that is, BA, kinetin, and 2ip). In tissue culture, these substances are associated with enhanced callus and shoot development.

Dioecious. Male and female flowers produced on separate plants.

Development. Changes in the form in the plant body caused by differentiation and growth.

Differentiation. Qualitative changes and differences that appear among cells, tissues, and organs during growth.

Electrophoresis. Separation of biological constituents based on their differential migration in an appropriate matrix subjected to an electric field.

Embryo. A plant form before germination.

Embryogenesis. Process of embryo initiation and development to an autotrophic plant.

Explant. Original part of the parent plant tissue that is introduced to *in vitro* conditions.

Free-living conditions. Natural or greenhouse conditions where the plantlets are removed from *in vitro* conditions and are transferred to soil mixtures. In such instances, plantlets must manufacture their own food supply for survival.

Gene. Unit of inheritance, located on the chromosome, which contributes to the control and maintenance of the cell.

Genetic variation. Occurrence of abnormal plantlets or cultures resulting from a genetic change during the tissue culture process.

Genotype. Genetic makeup of an organism.

Germination. Developmental stages associated with production of a plant from an embryo.

Growth. All quantitative changes during the life of an organism.

Haustorium. An absorbing organ, in date palms which is appressed to the endosperm and is part of the cotyledon.

Heterozygous. Genotype produced by the union of gametes containing different alleles.

Inflorescence. In palms, flower cluster or a spadix.

Inorganic salts. Mineral nutrients necessary for survival of biological organisms.

Isozyme. A multiple molecular form of an enzyme with similar or identical catalytic activities.

In vitro. A sterile, artificial environment, typically in glass vessels, in which cultured cells, tissues, organs or whole plants may reside.

In vivo. The natural conditions in which organisms reside.

Lateral bud. Axillary bud produced at the base of a leaf petiole.

Meristele. The vascular cylinder tissue in the stem.

Micropropagation. Rapid vegetative propagation of a plant using tissue culture technology, and usually beyond that obtainable in nature.

Monocotyledon. A flowering plant with one cotyledon in the embryo.

Morphogenesis. Study of the orgin of form.

Nutrient medium. A chemical formulation in which cells, tissues, and organs are grown *in vitro*; it provides nourishment and regulates morphogenetic events. Usually contains a source of inorganic salts, vitamins, carbohydrates, phytohormones, and, sometimes, complex addenda.

Organ. A tissue or group of tissues that constitute a morphologically and functionally distinct part of the organism (that is, leaf, inflorescence, root).

Organogenesis. Initiation of an organ structure, or the production of a plantlet *in vitro* through sequential nonsynchronized initiation of root and shoot structures connected by vascular tissue.

Parenchyma. A tissue composed of thin-walled, equal-sized cells; this tissue is usually meristematic and has storage capacities.

Phenotype. Outward visible appearance of an organism resulting from the interaction of genotype and environment.

Phytohormones. Plant hormones that include auxins, abscisic acid, cytokinins, gibberellins, and ethylene and other related substances. Phytohormones are chemical messengers that may pass through cells, tissues, and organs and cause biochemical, physiological, and morphological responses.

Plantlet. A plant with a distinct root and shoot system developed through tissue culture propagation either by embryogenesis or organogenesis.

Polyploid. Having more than two sets of chromosomes (2n).

Rachilla. A slender terminal division of a rachis within the spathe to which flowers are attached.

Rachis. Axis of a spadix.

Reculture. Transfer of the whole culture, cell, tissue, organ, or plantlet intact from one medium to another.

Shoot tip. Terminal bud of the plant. Consists of the apical meristem and the immediate surrounding leaf primordia.

Spathe. A broad sheathing bract enclosing the spadix.

Subculture. Division and transfer of a portion of a culture to fresh medium.

Suckering. Type of vegetative propagation where lateral buds grow out to produce an individual that is a clone of the parent.

Tissue. A group of cells of similar structure that performs a special function.

Tissue culture. A general term used to describe the development of plant cells, tissues, and organs cultured on a nutrient medium in sterile conditions.

Vitamins. Substances involved in the synthesis of coenzymes. It is necessary to add these substances to tissue culture preparations because they usually can not be produced *in vitro*.

GPO 792-095

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